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Genetic Variation Among Hawaiian Cultivars of *'uala* (*Ipomoea batatis*)

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ABSTRACT. Randomly Amplified Polymorphic DNA (RAPD) markers produced by five arbitrary 10-mer primers were used to determine the relationship between 12 cultivars of Hawaiian *Ipomoea batatas* (L.) Lam. Total DNA were extracted from leaves. Data was analyzed with multivariate principal component and cluster analysis. Leaves with a common shape were closely grouped. Yet within each group there still existed some degree of variability. The RAPD procedure provided a good estimate of genetic variation even within species *I. batatas* (L.) Lam., that can be used as a basis for further studies.

There are a number of items, such as linguistics, pottery, fish hooks, and tools, that can link cultures to each other. Plants can also be included as one of these artifacts. Genetic studies on *kalo*, *Colocasia esculenta*, presented the idea of two different cultivars based upon the amount of chromosomes. The migration route of one cultivar was traced from mainland Asia to Japan, to Indonesia, and onward to New Caledonia (Abbott 1992). In essence, the study not only arranged a migration pattern for the cultivar but also for the people carrying this *kalo*.

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Using Randomly Amplified Polymorphic DNA (RAPD), genetic relationships between cultivars can be analyzed and, similar to the information gathered form the *kalo* experiment, can be used to trace the movement of people and cultivar. The plant of this particular study is *Ipomoea batatas* (L.) Lam., or sweetpotato of Convolvulaceae. There are three different groups of sweetpotato, the Kumara, Batata, and Comote. All three of these groups are said to have origins in either central or south America. The Comote line was distributed to the Philippines, - Batata was taken from the Bahamas to Europe, and the Kumara was believed to enter the Pacific.

The sweetpotato, or *'uala* in Hawaiian, was found all throughout the Pacific. Depending on the culture, it was a minor crop such as on the island of Futuna, where it was used as an afternoon snack for women (Yen 1974). In other places such as the eight major Hawaiian islands it was a major crop, second only to *kalo*. Being a hardy plant, *'uala* was very important in the drier leeward *ahupua'as*, land divisions that usually stretched from valley ridges to the ocean. Tubers and leaves were cooked in an *imu*, an underground oven. Tubers were sometimes preserved by drying after cooking and taken on fishing trips, as done by the fishermen of *Ka'ū* (Abbott 1992). At other times the tubers were pounded into *poi 'uala*. Some parts of the plant were used medicinally. Old leaves and vines provided padding under floor mats (Wagner et al 1990).

The method of propagation was mainly through slips or cuttings. By placing the slips in a damp material such as *ti* (*Cordyline fruticosa*) leaves, roots were initiated to grow. After saying prayers to the gods and working the soil, about two or three slips were placed into mounds, or *pu'e*. This meant a limited number of genetic variability between crops. Corresponding mutation rates that cause changes in morphological expression were estimated to be low (Hernandez et al. 1964). As recorded by Handy, there were more than 230 cultivars in the Hawaiian Islands. The cultivars were classified by Hawaiians according to leaf shape, leaf color (top and bottom), vein color, stem color, tuber color (inside and out), and tuber shape.

This study is a preliminary analysis on Hawaiian *I. batatas* ,and coupled with existing data, such as morphological characteristics and genetic information of other sweetpotato cultivars in Oceania, could result in a better understanding of the cultivar's distributions throughout Polynesia. The step taken here is to provide base-line data on genetic variability and to establish relationships between varieties of Hawaiian sweetpotatos. Randomly Amplified Polymorphic DNA (RAPD) procedure was used. By using small, random sequence primers to start the DNA amplification process,

Table 1. *Ipomoea batatas* varieties from the Amy B. H. Greenwell Ethnobotanical Garden. Records of the Garden note that the names may not be the original names. Row 1 is located makai and rows 2 through 4 progressively mauka.

Sample Number	Variety Name	Accession Number	Garden Location	Leaf Shape
1	*'uala*	95.031	Mound 1	cordate
2	*'uala hehe nui*	95.036	Mound 2	cordate
3	*'uala hua moa*	95.035	Mound 3	cordate
4	*'uala palaa*	95.020	Mound 4	lobed
5	*'uala kilapaki*	95.037	Mound 5	lobed
6	*'uala papamu*	95.019	Mound 6	lobed
7	*'uala*	95.039	Mound 7	lobed
8	*'uala pulaa*		Mound 8	lobed
9	*'uala*		Row 1	cordate/ lobed
10	*'uala*		Row 2	lobed
11	*'uala*		Row 3	cordate
12	*'uala*		Row 4	lobed

the amplified product may show differences from one individual within a population to the next. This method has an advantage of being able to sample a large number of individuals very rapidly, is relatively inexpensive, and provides a good estimate of the genetic variation among the individuals. The major disadvantage is that statistical approaches to analyzing the data are not straightforward and are still being developed and tested.

Materials and Methods

Leaves of cultivars were obtained from the Amy B.H. Greenwell Ethnobotanical Garden located at Captain Cook, Hawai'i (Table 1). The material were placed in plastic baggies and stored at 4°C until ready for use.

Isolation of total cellular DNA and purification was done following the protocol of Morden et al. (1996). Leaves were washed and approximately 1.5 g weighed out. A grinding buffer consisting of 2% CTAB, 100mM Tris-HCL (pH 8), 1.4 NaCl, 20 mM EDTA, 0.2% beta-mercaptoethanol, and dH_2O was aliquoted into tubes and placed in a water bath, 60°-65°C. Samples were then ground in a pre-heated mortar with the buffer and some sea sand. Next, specimens were incubated in a water bath at 60°-65°C between 15-60 minutes. The material was then extracted once with SEVAG (24:1, chloroform : isoamyl alcohol) and centrifuged at 3000rpm for 10 minutes (Sorvall RT 6000D). The top liquid phase was transferred into a new tube. Using 2/3 volume of 2-propanol, DNA was precipitated. Again the samples were spun at 3000 rpm for 5 minutes to collect the precipitate. The liquid portion was poured off and the precipitate allowed to dry for a few minutes. A wash buffer of 76% ethanol, 10 mM ammonium acetate, and dH_20 was added to the pellet, which was then incubated for 10-60 minutes. Samples were spun, liquid poured off, and pellet dried for 5 minutes. For DNA purification, the pellet was then resuspended in 4 ml of TE and added to 3.9 g of cesium chloride and 50ml of ethidium. bromide. Ultracentrifugation using gradient density allowed DNA bands to be pulled. Ethidium bromide was removed with extractions of water saturated isobutanol and cesium chloride with at least the dialysis in TE. DNA was then stored at 4°C.

DNA concentrations were obtained by Shimadzu UV-1201 Spectrophotometer.

Table 2. Synthetic deoxyribonucleotides used as primers for amplification of sweetpotato DNA.

RAPD Primer	Nucleotide Sequence	# of Amplified Fragments
OPE-16	GGTGACTGTG	5
OPG-05	GTGAGACGGA	10
OPG-08	TCACGTCCAC	5
OPG-09	CTGACGTCAC	5
OPH-03	AGACGTCCAC	7

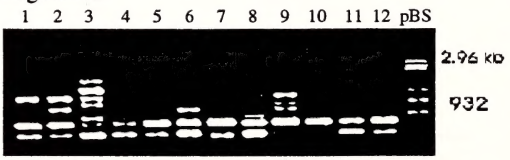
Primers were screened, and the best five were used for amplification (Table 2). PCR was carried out using a 25 µl reaction consisting of dH_20, primer in 100 mM stock, 1.25 mM of dNTPs, 10X PCR buffer, 25 mM $MgCl_2$, TAQ Polymerase (the latter three supplied by Promega), and approximately 12.5 mg/ml. Reactions were then run on a 1% agarose gel. Along with the DNA samples, pBS markers that had fragments between 2950 and 448 base pairs were run to help approximate the sweetpotato DNA fragment lengths.

Results and Discussion

RAPD markers ranged in length from 2100 to 390 base pairs depending on the primer and cultivar (Table 2 and Fig. 1). Markers for each cultivar were scored by either a "0" or a "1" , signifying absence or presence, respectively. A total of 25 bands were used for multivariate principal component and cluster analysis using Minitab for Windows Bit 32.

Graphing principal component 4 versus principal component 1, resulted in cultivars being partially arranged according to leaf shape (Fig. 2). Principal component four was mainly responsible for the separation of leaf types. Although similar leaf types were grouped together, there was still variability within each group possibly do to other factors that were not

Fig. 1. RAPD markers on 1% agarose gel. pBS=size marker used to determine size of fragments.



considered in this project, such as leaf pubescence, stem color, tuber color, etc. These other factors might have been considered in principal component one. Cordate and lobed leaves were again placed together when doing a cluster analysis (Fig. 3). The oddly shaped leaf of *'uala* sample 9 also appears to be the furthest related to everyone, this was also seen in the principal component graph. *'Uala hua moa*, or sample 3, in the cluster analysis is not grouped with the cordate leaves but rather more closely with sample 9. This could also be in part to other characteristics. Two ways in which this analysis could be more complete is to use more primers to acquire more RAPD markers and to including other morphological features of the cultivars. Despite those two drawbacks, RAPD analysis proved to be useful even to find variability within species.

Fig. 2 and 3. Genetic relationships among varieties of *'uala*. Fig. 2: Multivariate Principal Component Analysis. Fig. 3. Multivariate Cluster Analysis.

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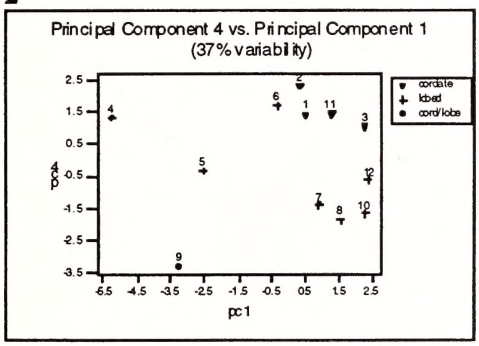

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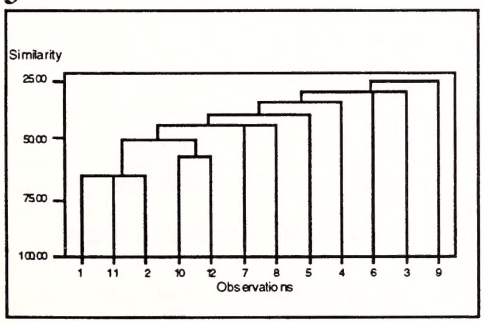

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***Ipomoea indica* (J. Burm.) Merr.**
(from Manual of the Flowering Plants of Hawai'i)

Effects of Population Fragmentation on Genetic Variation of *Haplostachys haplostachya*, an endangered Hawaiian Mint

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ABSTRACT. Randomly Amplified Polymorphic DNA (RAPD) are used to examine genetic variability within three sub-populations of the endangered mint *H. haplostachya* (Gray) St. John on the island of Hawai'i. Number of fixed genetic markers within the sub-populations were analyzed and show a genetic bottleneck within the small, disturbed sub-population at Pu'u Leilani. This study gives recommendations for future restoration efforts of *H. haplostachya* and illustrates the importance of genetic analysis prior to endangered species restoration.

Until a recent rediscovery on the Pohakuloa Plateau of Hawai'i, the endangered mint *Haplostachys haplostachya* (Gray) St. John (honohono), was thought to be extinct (USFWS). At one time a prevalent species in the mid-elevation (2000-3000 m) dry-forest between Mauna Loa and Mauna Kea on Hawai'i (Wagner et al. 1990), grazing pressure by feral ungulates, loss of habitat from military activity and conversion of forest to pastures have fragmented its range to small subpopulations. These remaining subpopulations are located within the military Pohakuloa Training Area (PTA) on several cinder cones and in the forested region of Kipuka Kalawamauna (USFWS).

An endemic genus within the Lamiaceae, *Haplostachys* once contained five species all thought to share a common ancestor with the genus *Phyllostegia* (Wagner et al. 1990). Four out of the five documented species are now extinct with the only extant representative, *H. haplostachya*, federally listed as endangered. Historically known from scattered collections at low elevation sites on Kaua'i and Maui, *H. haplostachya* are now restricted to a single population on the island of Hawai'i (Fig. 1). They appear as erect perennial herbs with white, typically lamiaceaeous flowers found in

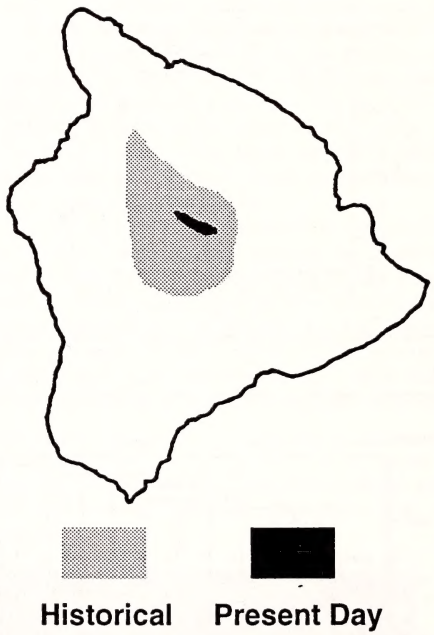

Fig. 1. Historical and Present Day Range of *Haplostachys haplostachya*.

dense terminal racemes (Wagner et al. 1990). Due to the historical rarity of *H. haplostachya* very little information on its biology is known (Wagner et al. 1990).

This study focuses on three distinct subpopulations: Pu'u Leilani, Pu'u Ka Pele, and Kipuka Kalawamauna. Until 1991, there had been no efforts to protect any of these subpopulations. At this time, the Army and the State Department of Land and Natural Resources (DLNR) came to an agreement that resulted in a four-foot fence being erected around the recently discovered population at the cinder cone Pu'u Ka Pele. Estimates of the number of individuals found at this cinder cone prior to fencing were about 3000 individuals; the population now has over 10,000 individuals, including numerous juveniles. The sub-population of Kipuka Kalawamauna also contains thousands of individuals and shows much reproductive activity. In contrast, Pu'u Leilani, a non-fenced cinder cone less than 2 km away, has 14 individuals remaining with no visible evidence of reproduction (either flowering or juveniles). However, even after the erection of fences, there are still several threats to the population. Feral ungulates (primarily goats, sheep and pigs) are commonly found at PTA, and these have been observed browsing the inflorescenses of *H. haplostachya* (Applet et al 1991a, 1991b), or digging up the soil. Military training poses threats to the plants from trampling during maneuvers as well as dust raised by passing vehicular traffic on the unimproved roads throughout the area. Fires also occur frequently at PTA as a result of various military activities; a 1992 fire destroyed several acres with numerous endangered species in different locations at PTA.

As a result of the intense habitat fragmentation and destruction of *H. haplostachya*, a once large, continuous population is now broken into small potentially inbreeding subpopulations. Inbreeding depression may be one of the most important genetic consequences of small population size (Lande 1988). The eventual effect of an increase in inbreeding within a population is reduced fitness and potential extinction due to increases in homozygosity, and therefore effectiveness of selection against recessive detrimental alleles (Barrett and Charlesworth 1991; Lande 1994; Lynch et al. 1995).

It is the purpose of this study to investigate the genetic diversity that remains among subpopulations of *H. haplostachya*, and determine what affect population fragmentation has had on its genetic structure. The results of this study could have significant impact on recovery efforts being planned for this and other species with small fragmented populations.

A method known as Randomly Amplified Polymorphic DNA (RAPD) was chosen in order to determine the relative amount of genetic variation within and among the subpopulations of *H. haplostachya*. This method, introduced by Williams et al. (1990), uses single primers of arbitrary nucleotide sequence (8-10 base pairs in length) to amplify a set of fragments of nuclear DNA. Amplification products (usually 2-10 fragments per primer) are then visualized by electrophoresis in ethidium bromide-stained agarose gels. Polymorphisms among the amplification products are common and can be useful as dominant genetic markers inherited in a mendelian fashion (Williams et al. 1990; Howland and Arnau 1991; Hadrys et al. 1992; Roderick 1996).

In the few years since the potential of this procedure in population studies was recognized, the RAPDs method has become a popular method of genetic mapping in plant and animal breeding studies (Dunemann et al. 1994, Rieseberg et al. 1994), discerning hybrid origin (Arnold et al. 1991; Rieseberg and Gerber 1994), determining rates of outcrossing in plant populations (Fritsch and Rieseberg 1992), and detection of gene introgression (Arnold et al. 1991; Waugh et al. 1992; Orozco-Castillo et al.

MATERIALS AND METHODS

Plant Collection. Under section 10(a)(1)(A) of the Endangered Species Act, 16 U.S.C. 1531 et seq. a collecting permit (#PRT-811049) was issued by the U.S. Department of the Interior and leaf tissue from plants of *H. haplostachya* was collected from three subpopulations at PTA. A single leaf was removed from plants spaced every 10 m along a vertical transect from the top to bottom of Pu'u Ka Pele (total of 16 plants sampled). A subpopulation in Kipuka Kalawamauna along Bobcat trail was sampled by arbitrarily selecting a leaf from plants at least 5 m apart (21 plants sampled). A leaf from all individuals at Pu'u Leilani were sampled (14 plants sampled). Leaf samples were placed into ziplock plastic bags and kept chilled, but not frozen, until the DNA extraction (Table 1). A single voucher from

Table 1. Number of individuals sampled and sampling method for each subpopulation.

Subpopulation	No. Indiv. Sampled	Sampling Method
Pu'u Leilani	14	All individuals
Pu'u Ka Pele	16	Transect, 10 m intervals
Kipuka Kalawamauna	21	Random, 5 m apart

each site was collected (*Morden 1341, 1406, 1414*) and will be on deposit at BISH.

DNA Extraction and Amplification. DNA from each of the 51 individuals was extracted according to methods outlined in Morden et al. (1996). Approximately 25 ng of DNA was subjected to thermocycling amplification using 25 µl reactions in Randomly Amplified Polymorphic DNA (RAPD) under the following conditions: 0.2mM each of dATP, dCTP, dGTP, and dTTP, 1X Taq polymerase buffer, 1.5mM $MgCl_2$, 2mM random 10-mer oligonucleotide primer (Operon Technologies, Alameda, CA), and ca 1 unit Taq Polymerase (Promega, Madison, WI). Each reaction was overlaid with two drops mineral oil. Amplifications were performed in a Hybaid OmniGene Temperature Cycler programmed for one cycle at 94°C for 3 min., 35°C for 30 sec., and 72°C for 2 min., followed by 43 cycles at 95°C for 45 sec., 35°C for 30 sec., and 72°C for 2 min., and a final cycle of 95°C for 45 sec., 35°C for 30 sec., and 72°C for 6 min. Amplified products were electrophoresed on 1.5% agarose gel in a 0.5X tris-borate-EDTA (TBE) buffer, and the DNA bands visualized by ethidium bromide staining. Size of amplified products were estimated by using a marker produced from pBS plasmid (Stratagene, La Jolla, CA) digested with a combination of restriction enzymes to produce fragments in a size range of 0.448 to 2.96 kb.

RAPD Analysis. DNA from six indivuals, two from each sub-population, were subjected to amplification with a total of 48 RAPD primers. Control samples containing all reaction material except DNA were used, in order to check that no self-amplification or DNA contamination occurred. Amplification products of each primer were assessed. Only the 23 primers producing consistent amplification of well defined, brightly staining bands were used in further amplifications of DNA from all 51 individuals (Table 2). Final amplifications were repeated to assure that the amplification products (ranging from as few as four to as many as nineteen from one primer) being scored were repeatably visible and not artifacts of the particular amplification run. Bands were labelled by the primer and approximate size of the band and scored as fixed present, fixed absent or variable within each population.

Table 2. Primer, primer sequence, and corresponding number and size range of scored amplification products.

Primer	Nucleotide Sequence	Scored Products	Size Range (bp)
OPA-1	CAGGCCCTTC	6	580-1900
OPA-5	AGGGGTCTTG	9	380-2000
OPA-7	GAAACGGGTG	13	360-2850
OPA-8	GTGACGTAGG	6	630-995
OPA-9	GGGTAACGCC	6	580-1200
OPA-10	GTGATCGCAG	8	420-2100
OPA-12	TCGGCGATAG	4	300-2050
OPA-13	CAGCACCCAC	13	450-2000
OPA-16	AGCCAGCGAA	11	380-1800
OPA-18	AGGTGACCGT	11	525-2150
OPA-20	GTTGCGATCC	5	1100-1900
OPB-2	TGATCCCTGG	11	420-2350
OPB-3	CATCCCCCTG	2	430-867
OPB-4	GGACTGGAGT	3	575-1000
OPB-17	AGGGAACGAG	9	380-1500
OPC-4	CCGCATCTAC	11	600-2300
OPC-7	GTCCCGACGA	4	620-1450
OPC-8	TGGACCGGTG	8	480-2500
OPC-12	TGTCATCCCC	5	700-1650
OPC-13	AAGCCTCGTC	11	460-2350
OPC-15	GACGGATCAG	5	660-1400
OPC-18	AGGTGACCGT	9	540-2000
OPC-19	GTTGCCAGCC	7	580-1250

RESULTS

After PCR amplification with all 24 RAPD primers with DNA from all 51 individuals, a total of 177 genetic markers, representing putative genomic loci, were examined. Of the 177 markers examined, 63 were fixed present across all individuals and were therefore uninformative for further analysis. However, 114 markers were variable in at least on of the three sub-populations (Table 3).

Table 3. Number of primers screened versus surveyed. Number of genetic markers examined, including markers that were fixed present across all individuals as well as informative markers with variation between and among populations.

Statistic	Total
Primers Screened	48
Primers Surveyed	21
Total Markers Scored	177
Markers Fixed Present in All Individuals	63
Informative Markers	114

Further analysis of the variability of these markers within the sub-populations show Pu'u Leilani to have 39 of the 114 informative (34%) markers fixed either present or absent across all 14 individuals. Pu'u Ka Pele exhibits only 29 of the 114 (25%) markers fixed across all individuals, while the Kipuka Kalawamauna sub-population has only 13 (11%) fixed markers (Table 4).

DISCUSSION

The percentages of informative (variable within at least one population) genetic markers (n=114) that were fixed present within each sub-population can be viewed as an inverse indicator of genetic variability within that population. A population with high heterozygosity among individuals will have fewer markers fixed (either present or absent) across all of its individuals, than a population with high homozygosity among individuals. The results of this study show that the Pu'u Leilani sub-population, with only 14 individuals, has undergone a genetic bottleneck in comparison to the other two sub-populations which both exhibit fewer fixed genetic markers, and therefore greater genetic variability. This comparison is appropriate given historical evidence that suggests that these three sub-populations were at one time continuous with one another. The results also show Pu'u Ka Pele, the recently fenced and recovering population to have an intermediate amount of variability, while the large Kipuka Kalawamauna population exhibits the most variation within a sub-population. These results are supported by the lack of observed flowers or juveniles at the Pu'u Leilani site, and the recent recovery from 3000 to 10,000 individuals at the Pu'u Ka Pele population. It would seem that the Pu'u Ka Pele population is recovering from a slight but recent bottleneck, while the Kipuka Kalawamauna, the less historically disturbed of the three sites is more representative of the original continuous population.

These results suggest that habitat fragmentation does have a significant effect of limiting genetic variability within sub-populations of *H. haplostachya.* This research also illustrates the importance of genetic structure analysis on existing populations of endangered species prior to restoration attempts. Therefore the following recommendations are made for future resoration efforts within the historic range of *H. haplostachya*: 1) Seed source should be harvested from Kipuka Kalawamauna because of the greater genetic variability contained within that population, and 2) Kipuka Kahekili (near Waikii Ranch) would be the most suitable outplanting site because of habitat suitability and low disturbance.

Table 4. Number of total markers screened and surveyed, fixed markers, and variable markers for the *H. haplostachya* sub-populations.

	Pu'u Leilani	Pu'u Ka Pele	Kipuka Kalawamauna
No. Individ. in Pop.	14	ca. 3000[a]	>1000
Reproductive Activity	Non-Flowering, No Juveniles	Abundant Flowering, Many Juveniles	Abundant Flowering, Many Juveniles
No. Fixed Markers	39	29	13
No. Variable Markers	66	79	93

[a]Number of individuals estimated at time cindercone was fenced. Subpopulation now estimated at ca 10,000 individuals.

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Identification of *Dubautia paleata* × *raillardioides* Hybrids Using RAPD Markers

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ABSTRACT.- Hybridization appears to be a common phenomenon in the Hawaiian silversword alliance, where 35 naturally occurring hybrid combinations have been reported between species in the genera *Agyroxiphium*, *Wilkesia*, and *Dubautia*. Hybridization has been suspected to occur between *Dubautia paleata* and *D. raillardioides* based on the occurrence of morphological intermediates between the two taxa; however, no research has yet been done to document hybridization between them. Random amplified polymorphic DNA (RAPD) markers were used to determine if 6 of these morphologically intermediate plants are, in fact, hybrids. Five primers were used to generate 64 markers, 5 of which were specific for *D. raillardioides*, and 4 of which were specific to *D. paleata*. All 4 bands specific to *D. paleata* and 2 bands specific to *D. raillardioides* were present in the hybrids. In PCA and cluster analysis, the hybrids cluster between *D. paleata* and *D. raillardioides*, although the hybrids appear more closely aligned to *D.* paleata. This data suggest that the putative hybrids are hybrids between *D. paleata* and *D. raillardioides*, and show introgression with *D. paleata*.

The evolutionary importance of hybridization is illustrated by the estimation that perhaps 70% of all flowering plants species have arisen through hybridization (Whitham et al. 1991). Recent studies are addressing new ideas and approaches concerning its mechanisms and consequences (Rieseberg 1995). Perhaps the most well-studied hybrid plant system is the Louisiana iris complex. Arnold and his colleagues have found the F_1 hybrid formation is Louisiana irises is rare (Arnold 1993); intraspecific pollen is a superior competitor to interspecific pollen and has a greater capacity to produce mature seeds (Arnold et al. 1993), and the hybrid population represents advanced generations that have a large degree of introgression (Arnold et al. 1990; Cruzan and Arnold 1993). Studies which assess key characteristics of hybrid populations will lead to a better understanding of evolutionary consequences of hybridization. Probable roles of hybridization may be to increase the genetic variability in a population through recombination, and to form new species through stabilization of the hybrid population (Rieseberg 1995; Carr 1995). Hybrids may be able to exploit new habitats unoccupied by either parent species (Lewontin and Birch 1966; Arnold 1994).

In geologically unstable and geographically isolated floras such as in Hawaii, hybridization may be critical in the maintenance of colonizing species, and in the exploitation of new habitats (Carr 1995). Founding populations have a limited gene pool, and recombination through hybridization represents a way to maximize genetic variation in small populations (Rieseberg 1995; Carr 1995). Many species in the Hawaiian flora are, in fact, noted for their lack in internal barriers to hybridization (Gillet 1972; Carr 1995). The greater degree of genetic variation that hybridization affords populations may also facilitate a more rapid advance into new habitats (Gillet 1972; Cruzan and Arnold 1993). Because varying habitats are closely juxtaposed in Hawaii, the

probability that a hybrid may reach a habitat where it might become stabilized is increased (Carr 1995).

The occurrence of hybridization is well-documented in Hawai'i (Smith et al. 1962; Gillet 1972; Carr 1995). Notable examples of hybridization in the Hawaiian flora include *Scaevola*, *Cyrtandra*, and the silversword alliance. For example, 67 natural hybrid combinations have been found between *Cyrtandra* species (Smith et al. 1962). Within the silversword alliance, 35 naturally occurring hybrid combinations have been documented (Carr 1985). Furthermore, intergeneric hybridization between *Argyroxiphium sandwincense* ssp. *macrocephalum* and *Dubautia menziesii* occurs (Carr 1995).

The morphological and ecological diversity of the silversword alliance stem from a single introduction (Carr and Kyhos 1981), and have resulted in a radiation comprising 28 endemic species in the genera *Argyroxiphium*, *Wilkesia*, and *Dubautia* (Carr 1985). Species in the silversword alliance are found in habitats as diverse as bogs, wet forests, and alpine deserts (Carr 1985). *Dubautia* is the most speciose genus in the alliance, with 21 species distributed across 6 of the main islands, the majority of species being single island endemics (Baldwin and Robichaux 1995). In *Dubautia*, hybridization is a frequent occurrence (Carr and Kyhos 1981). Carr and Kyhos (1981) studied 7 different *Dubautia* hybrid crosses for cytogenetic analysis. Carr (1985) lists a remarkable number of collected hybrids for several *Dubautia* species. For example, *D. paleata* is known to hybridize with *D. imbricata* spp. *acrononaea*, *D. laxa* spp. *laxa*, and *D. waialealae* (Carr 1985).

D. paleata, a Kauai endemic, occurs in open boggy areas of the Alakai Swamp at elevations ranging from 1100 to 1550 m (Wagner et al. 1990).). *D. paleata* is a small dense shrub, 0.3 to 2.5 cm in length. Its leaves are pubescent, opposite or ternate, lanceolate, and 3 to 20 cm in length (Wagner et al. 1990). *D. raillardioides*, a closely related species (Baldwin and Robichaux 1995), and is also present in the Alakai Swamp. However, its habitat is in wet forests at the margins of bogs, at elevations from 600 to 1375 m (Wagner et al. 1990). *D. raillardioides* is an understory shrub, up to 3 m tall, with a lax, partly decumbent branching shape. Its leaves are ternate, glabrous, lanceolate, 9-25 cm in length, with margins conspicuously toothed form the apex to halfway to the base (Wagner et al. 1990).

In marginal areas between bogs and wet forests in the Alakai Swamp region, large leafed plants with fewer trichomes than typical for *D. paleata* are observed (Wagner et al. 1990; Roderick, pers. comm.). Because morphology of these plants seem intermediate between *D. paleata* and *D. raillardioides*, these individuals appear to be hybrids between these two species. Hybrids between *Dubautia* species are commonly intermediate in the foliar characters (Carr and Kyhos 1981; Kim 1987), although this is not necessarily true of hybrids in general (Rieseberg 1995). Also, this marginal habitat appears to be intermediate between the habitats of *D. paleata* and *D. raillardioides*; thus, intuitively it seems possible that hybrids may occur here.

Here, we attempt to clarify the identity of these individuals using RAPD markers. These 'morphological intermediates' may either be of hybrid origin, or represent larger morphologies of *D. paleata*. This determination is an important first step in assessing the dynamics between these two *Dubautia* species.

Genetic analysis using RAPD markers have been useful in determining hybridization in several taxa, including Louisiana irises (Cruzan and Arnold 1993), Catalina Island mountain mahogany (Rieseberg and Gerber 1995), and ×*Margyrancaena skottsbergii* (Crawford et al. 1993). The use of RAPD markers is effective because many markers can be generated, and little DNA is required (Crawford et al. 1993).

Materials and Methods

Plant material. Plant samples were collected from the Alakai Swamp on the summit plateau of the island of Kauai, Hawaii, at approximately 1250 m in elevation. Leaves of 10 plants suspected to be of hybrid origin were collected from a putative hybrid zone located close to the end of the boardwalk trail, where the trail begins to loop northward. This area is a transition zone between the bog and wet forest. These tentative hybrids appeared to have vegetative parts that are of intermediate size and pubescence between *D. paleata* and *D. raillardioides*. 7 samples of *D. paleata* were collected along the boardwalk trail in the flat, boggy areas of the swamp, at least 10 m away from the putative hybrid zone. 9 samples of

Table 1. Species-specific bands (in kb) present *D. paleata* and *D. raillardioides*, and their occurrence in putative hybrid plants.

Primer	*D. pal.*	*D. pal.* × *rail.*	*D. rail.*
OPG-6	–	–	1.55
	–	1.40	1.40
	0.56	0.56	–
OPG-9	0.96	0.96	–
OPG-11	–	–	2.1
	1.4	1.4	–
OPG12	2.6	2.6	–
	–	1.4	1.4
OPI-13	–	–	0.55

D. raillardioides were collected at the beginning of the boardwalk trail at the margin of wet forest.

DNA Extraction and Amplification. Total nuclear DNA was extracted using a method based on Doyle and Doyle's (1987) modification of the CTAB isolation procedure and is described in Morden et al. (1996).

10-mer random primers (Operon) were used for amplification of *Dubautia* DNA. RAPD amplifications were carried out in 25 µl reaction mixtures containing 1× Fisher buffer, 2.0 mM $MgCl_2$, 0.1 mM each of dATP, dTTP, dCTP, and dGTP, 8µM primer, 0.75 units of Taq DNA polymerase, and approximately 25 ng of DNA. The reaction mixture was overlaid with approximately 25 µl of mineral oil. Amplification was performed in a Hybaid OmniGene thermocycler with the following conditions: one cycle of 94°C for 3 min., 35°C for 30 sec., 72°C for 2 min.; 43 cycles of 94°C for 45 sec., 35°C for 30 sec., 72°C for 2 min.; a final cycle of 94°C for 45 sec., 35°C for 30 sec., 72°C for 6 min., for one cycle. Amplification products were separated on 1.5% agarose gels. Ethidium bromide was included in the gel and electrophoresis buffer (0.5× TBE), and products were visualized and photographed under ultraviolet light.

Data analysis. For each individual, bands from all markers were scored as either absent (0) or present(1). The amplification products were inspected for bands which were restricted to either *D. paleata* or *D. raillardioides* and also found in the putative hybrids. Multivariate statistics of principal components analysis (PCA) and cluster analysis were performed for further data analysis. Minitab statistical software was used for multivariate analyses.

Results

Seven individuals of *D. paleata*, six individuals of *D. raillardioides*, and six putative hybrids consistently amplified with 5 primers: G6, G9, G11, G12, and I13. The five primers produced a total of 64 bands, of which 9 were relatively species-specific (Table 1). 5 of these bands were restricted to *D. raillardioides*, and the other 4 were restricted to *D. paleata*. The putative hybrids exhibited all four markers restricted to *D. paleata*, and 2 markers restricted to *D. raillardioides*.

PCA shows that *D. raillardioides* is a distinct population from *D. paleata* and the putative hybrids (figs. 1 and 2). Individuals of *D. paleata* appear to be highly variable in relation to each other; thus, loosely clustered. The putative hybrids appear to be somewhat clustered together and interspersed in the *D. paleata*; however, a weak segregation between the putative hybrids and a majority of *D. paleata* individuals is evident along the x-axis (PCA 1). Also, the putative hybrids segregate between *D. raillardioides* and the edge of *D. paleata*, indicating that *D. raillardioides* is more similar to the putative hybrids than it is to *D. paleata*.

For the cluster analysis, 28 out of the 64 markers that accounted for most of the variation as determined from PCA were used. These results also indicate that *D. raillardioides* form a distinct cluster from *D. paleata* and the putative hybrids (fig. 3). Individuals of *D. paleata* and the putative hybrids are more closely aligned. However, it is clearly evident that the putative hybrids form a distinct group from *D. paleata*, and that the putative hybrids again cluster in between *D. raillardioides* and *D. paleata*, indicating that the putative hybrids are genetically intermediate between these two species.

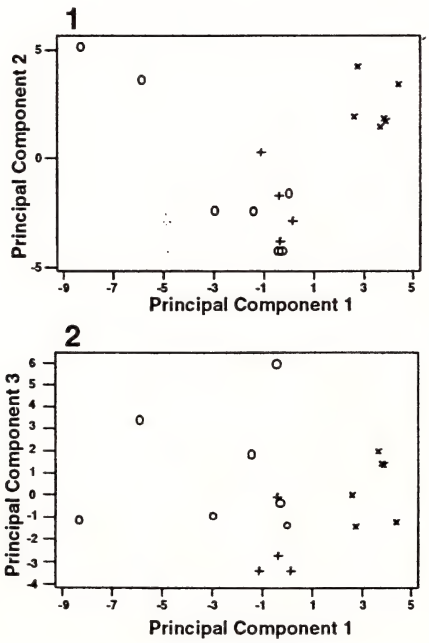
Discussion

Based on this preliminary data, the putative hybrids identified using morphological characteristics appear to be of hybrid origin. The putative hybrids contain bands which were specific to both parental species, indicating that

the hybrids contain genetic material from both species. PCA and cluster analysis also confirm that the pututive hybrids are intermediate between *D. paleata* and *D. raillardioides*. Because the hybrids display more markers specific to that of *D. paleata*, and that in PCA and cluster analysis, the hybrids are more closely aligned to *D. paleata*, the individual hybrids sampled may represent advanced generations which show introgression into *D. paleata*. The greater degree of separation between *D. raillardioides* and the hybrids, than between *D. paleata* and the hybrids shown in the PCA and cluster analysis is not surprising given the spatial distribution of the populations sampled. The populations of *D. paleata* and the hybrids which were sampled were within 10 m to 50 m of each other. The population of *D. raillardioides* that was sampled was approximately 1.6 km from the hybrid zone. Thus, one would expect to see a greater degree of similarity between the samples of *D. paleata* and the hybrids than between *D. raillardioides* and the hybrids. If samples of *D. raillardioides* were taken from closer to the hybrid zone, then perhaps the data would show a greater degree of similarity between *D. raillardioides* and the hybrids. A population of *D. raillardioides* within 10 m of the hybrid zone was not found in a cursory search (C. Ewing, per. comm.). However, this search was not thorough, and the intertwining habit of *D. raillardioides*, along with the topography of the region would have made any individuals hard to find.

Because of the small number of hybrids and parental species sampled, and the small number of markers generated, it is not possible to make any detailed assessment of the population dynamics found within this group in this study. However, the great degree of similarity between the hybrids and *D. paleata* does suggest that the hybrids are not F_1 hybrids, and that this population is introgressing with *D. paleata*. In order to more fully assess the characteristics of this hybrid zone, several studies may be undertaken to find a) the population of *D. raillardioides* that contribute to the hybrid zone b) the frequency of F_1 hybrid formation and c) the degree of recombination that occurs between *D. paleata* and *D. raillardioides* due to hybridization.

Given the phylogenetic closeness of *D. paleata* and *D. raillardioides*, the close juxtaposition of their habitats in the Alakai Swamp, and *D. paleata*'s propensity to hybridize, it is not surprising that these two taxa have hybridized in at least one region of the Alakai Swamp. This study offers another example of the lack of genetic barriers within the Hawaiian flora.



Figs. 1 and 2. Principal components analysis of *D. paleata* (o), *D. raillardioides* (x), and hybrids (+). 1. PCA 1 and PCA 2 accounting for 40.3% of the total variation. 2. PCA 1 and PCA 3 accounting for 33.5% of the total variation.

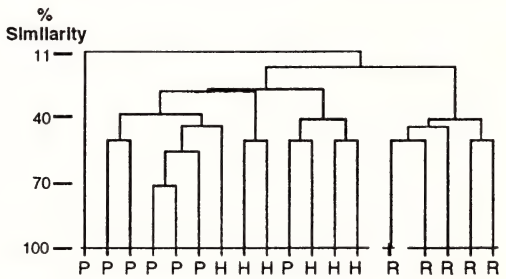


Fig. 3. Cluster analysis of *D. paleata* (P), *D. raillardioides* (R), and putative hybrids (H).

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Dubautia raillardioides Hillebr.

(From Manual of the Flowering Plants of Hawai'i)

"Sierra Club Legal Defense Fund" Changes Name to "Earthjustice Legal Defense Fund"

On June 14, 1997, the *Sierra Club Legal Defense Fund* announced that it is changing its name to *Earthjustice Legal Defense Fund*, effective immediately. The decision was made by unanimous vote of the organization's Board of Trustees meeting in Tiburon, California, on June 14. Earthjustice Legal Defense Fund is a non-profit, public interest, environmental law firm that employs approximately 50 attorneys in nine offices across the country. While its name is changing, the Legal Defense Fund's mission of protecting people and resources by enforcing and strengthening our environmental laws continues as strong as ever. The group has its headquarters in San Francisco and field offices in Juneau, Honolulu, Seattle, Denver, New Orleans, Tallahassee, Washington, D.C., and Bozeman, Montana.

The Mid-Pacific office, located in Honolulu, opened in 1988 with a grant from the MacArthur Foundation. The Fund has successfully litigated matters under state and federal laws, including the Endangered Species Act, Clean Water Act, National Environmental Policy Act, State Water Code, and Hawai'i's "Sunshine" Law. In addition to legal services to the public, which are provided free of charge, the Legal Defense Fund assists its clients in community organizing, lobbying, and media relations. Since 1995 the Mid-Pacific office has also sponsored the Ahupua'a Action Alliance, a statewide coalition of over 65 environmental and Hawaiian organizations, which was created as part of the Legal Defense Fund's Marine Biodiversity Project.

The Legal Defense Fund represented the Hawaiian Botanical Society in litigation that resulted in the listing of over 180 candidate Hawaiian plants as threatened or endangered. Currently, the Legal Defense Fund represents the Hawaiian Botanical Society, Conservation Council for Hawai'i, and Sierra Club in a legal action to compel the U.S. Fish and Wildlife Service to designate critical habitat for these plants, as required by federal law.

"We are proud to continue to represent grass-root efforts to protect Hawai'i's special environment and culture." said Paul Achitoff, managing attorney for the Mid-Pacific of fice. In addition to Achitoff, the current staff in Hawai'i include associate attorney David Henkin, resource analyst Marjorie Ziegler, Ahupua'a Action Alliance coordinator Lynette Cruz, part-time resource analyst/Alliance staff Kat Brady, office manager Kim Ramos, litigation secretary Everett Ching, secretary/receptionist Linda Shapin, and office assistant Karen Miles.

***Capparis sandwichiana* DC**
(From Manual of the Flowering Plants of Hawai'i)

Minutes of the Hawaiian Botanical Society

April Meeting

• The April 7 meeting of the Hawaiian Botanical Society was called to order by Wisteria Loeffler, President.

• The minutes were approved as read.

• **Treasurer's Report.** Ron Fenstamacher read the treasurer's report.

• **Membership Report.** Alvin Yoshinaga, membership chair, announced five new members.

• **Old Business**
  • Botanical Society's garage sale netting $430 and the announcement of the Science Fair winners.

• **New Business**
  • The Earth Day chair, Orlo Steele, asked for volunteers to help staff the society's exhibit.

  • The annual plant raffle for the May meeting was announced.

  • The April hike is combined with a *Miconia* search with Pat Conant as trip leader.

• **The Plant of the Month** talk was Tahitian Flora Endangered by *Miconia calvescens*, given by Jean-Yves Meyer, Minister of Health and Research at Tahiti.

• **Guest Speaker.** The speaker for April was Kenneth Nagata of US Department of Agriculture, talking about the USDA International Pre-Clearance Program: Dutch Flower Bulb Inspections.

***Rubus macraei* A. Gray**

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- ***A Preliminary Study on the Origin of the Hawaiian Endemic Genus* Hibiscadelphus *(Malvaceae). D. Weniger and C. W. Morden***
- ***Common Native Hawaiian Plants Worthy of Cultivation. J. K. Obata***
- ***Growing Native Hawaiian Plants. J. K. Obata***

Do you have something you would like to contribute to the *Newsletter*? All contributions are welcome. They may be technical articles related to on-going research, comments about current events, or field observations you may have made from a recent expedition. Contributions may be sent to the *Newsletter* Editor via manuscript, disc (please provide hard-copy also), or E-mail at:

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***Abutilon menziesii* Seem. and *Abutilon sandwicense* (Degener) Christoph.**
(From Manual of the Flowering Plants of Hawai'i)

***Asplenium contiguum* Kaulf.**

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